Gene Transfer into Cardiac Myocytes in vivo

Leslie A. Leinwand and Jeffrey M. Leiden

The ability to express recombinant genes in cardiac myocytes in vivo holds promise for the treatment of a number of inherited and acquired diseases of the cardiovascular system. Several groups have demonstrated recently that plasmid DNA is taken up and expressed in cardiac myocytes following injection into the left ventricular wall in vivo. Recombinant genes introduced into cardiac myocytes by this technique are expressed for at least 6 months after injection, and appear to be regulated normally by humoral signals. In addition to its potential for somatic gene therapy, this method should prove useful for studies of transcriptional regulation in the heart. (Trends Cardiovasc Med 1991; 1:271–276)

Somatic gene therapy can be defined as the ability to program the expression of recombinant genes in non-germ-line cells of a recipient host. Recent advances in molecular biologic techniques, including the cloning of a large number of eukaryotic and prokaryotic genes, and the development of viral and chemical gene delivery systems have brought us to the threshold of a new era of somatic gene therapy [reviewed by Friedmann (1989), Swain (1989), and Nabel and Nabel (1991)]. A wide range of gene therapy experiments are being pursued in animal systems (Lemischka et al. 1986; Hock and Miller 1986; Garver et al. 1987; Palella et al. 1988; Dichek et al. 1989; Nabel et al. 1990; Wilson et al. 1989), and the first trials of human gene therapy have been initiated recently in patients with inherited enzyme deficiencies and malignancies (Rosenberg et al. 1990).

The ability to program recombinant gene expression in cardiac myocytes in vivo holds promise for the treatment of a number of disorders of the cardiovascular system. Potential approaches to somatic gene therapy in the heart can be divided into two groups. Indirect gene therapy methods involve the introduction of recombinant genes into recipient cells in vitro, followed by the transplantation of these transduced cells back into an appropriate host organism. Such indirect approaches utilizing cardiac myocytes have been restricted severely by our inability to grow and manipulate these cells in vitro. Normal neonatal and adult cardiac myocytes display a very limited life span in tissue culture (Zak 1974), and only one cardiac cell line has been described (Steinhelper et al. 1990). Because this cell line was derived from an SV40 T-antigen transgenic animal and causes tumors when injected into nude mice, it may not represent an ideal reagent for studies of cardiac somatic gene therapy.

A second type of approach to somatic gene therapy in the heart, direct gene transfer, involves the introduction of recombinant genes into recipient cells of the intact heart in vivo, without the need to remove the cardiac myocytes from the host organism. Any such method of direct gene therapy must fulfill a number of requirements, including an efficient technique for delivering DNA into the target tissue, the ability to program stable expression in the correct cell type, and a minimal risk of harmful side effects to the recipient organism. Thus far, three types of approaches have been used to deliver DNA into an intact organ. The first involves the use of biochemical delivery systems in which the DNA is complexed with a variety of materials, including liposomes (Wang and Huang 1987; Felgner et al. 1987; Nicolau et al. 1983; Nabel et al. 1990), polylysine–glycoprotein (Wu et al. 1989), and a calcium–phosphate precipitate. The second approach involves the use of viral transduction systems in which the exogenous gene is incorporated into a recombinant viral genome that is then used to infect cells within the organ [for the cardiovascular system, see Nabel et al. (1990)]. The third method, which is rather remarkable for its simplicity, involves the direct injection of purified DNA into adult tissues. This approach was first demonstrated in mouse skeletal muscle (Wolff et al. 1990). Recently, several groups have demonstrated that this type of direct injection approach can be used to program recombinant gene expression in adult rodent cardiac myocytes in vivo (Lin et al. 1990; Acsadi et al. 1991; Kitsis et al. 1991). This review describes the results of these initial experiments, with particular emphasis on the efficiency, stability, and safety of recombinant gene expression in the heart. In addition, we address the question of whether such recombinant genes are regulated normally in response to physiologic stimuli following their introduction into cardiac myocytes in vivo.

• Efficiency and Stability of Gene Transduction into Cardiac Myocytes in vivo

Wolff et al. (1990) were the first to demonstrate that recombinant genes

Leslie A. Leinwand is at the Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461; and Jeffrey M. Leiden is at the Howard Hughes Medical Institute and the Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109, USA.

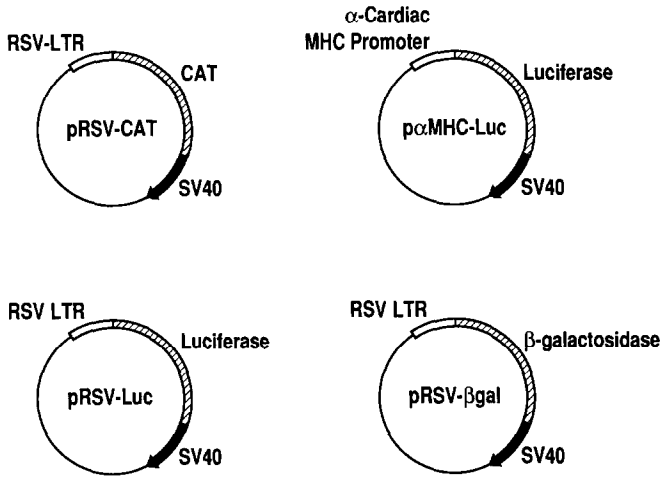


Figure 1. Reporter plasmids used for cardiac injections. Schematic representations of the reporter plasmids used in studies of recombinant gene expression in cardiac myocytes in vivo. The Rous sarcoma virus long-terminal repeat (RSV LTR) containing the viral promoter and enhancer sequences and the 613 bp α myosin heavy-chain promoter (α-cardiac MHC promoter) are shown as *open boxes*. The chloramphenicol acetyltransferase (CAT), luciferase, and β-galactosidase reporter genes are shown as *hatched boxes*. The SV40 sequences containing the polyadenylation site and small t intron are shown as the *solid arrow*.

could be taken up and expressed in a small percentage of murine skeletal myofibers following direct intramuscular injection of purified DNA or RNA. The mechanisms underlying nucleic acid uptake by skeletal muscle remain unclear, but expression of the recombinant genes was shown to be stable for at least 2 months following injection. Three laboratories have now used similar direct DNA injection methods to express recombinant genes in the heart (Lin et al. 1990; Acsadi et al. 1991; Kitsis et al. 1991). As shown in Figure 1, these experiments have involved the use of three different reporter genes: chloramphenicol acetyltransferase (CAT), luciferase (Luc), and β-galactosidase (βgal). These reporter genes were chosen because none of them are normally expressed in cardiac myocytes, and because each can be easily assayed in vitro. CAT is a bacterial enzyme that is easily quantified by thin-layer chromatography. Luciferase is a firefly enzyme that generates light in the presence of adenosine triphosphate and a specific substrate. It has the advantage of being more sensitive than CAT so that lower levels of expression can be detected. β-Galactosidase is a bacterial enzyme that has the advantage of being assayable both histochemically (in the presence of an appropriate substrate, it catalyzes a reaction that stains cells blue) and biochemically. Thus, it can be used to identify cells expressing recombinant gene products in vivo. The ability to identify β-galactosidase expression histochemically is particularly important in the heart, in which <50% of the cells are cardiac myocytes.

Both viral and cellular transcriptional regulatory sequences have been used in the initial studies of recombinant gene expression in cardiac muscle. The Rous sarcoma virus long-terminal repeat (RSV LTR), which contains both promoter and enhancer sequences, has proven to be a useful transcriptional regulatory element, because previous work has shown that it programs high-level transcription in cardiac myocytes both in vitro (Gustafson et al. 1987) and in vivo (Overbeek et al. 1986). Similarly, the α cardiac myosin heavy-chain (αMHC) gene is expressed in a cardiac-specific manner and is positively regulated by thyroid hormone (Izumo et al. 1986; Lompre et al. 1984). The promoter and flanking sequence have been shown to be active in transfected myocytes and to be responsive in vitro to thyroid hormone (Gustafson et al. 1987; Tsika et al. 1990). Thus, this promoter is useful for studies of the humoral regulation of recombinant genes in cardiac myocytes in vivo.

The direct DNA injection methods used in the three studies were similar. Each of the groups utilized a narrow (27–30 gauge) needle to inject solutions of purified supercoiled plasmid DNA. The vehicle used for DNA injection appears to be relatively unimportant, with successful gene transfer resulting from the use of Opti-MEM, normal saline, and 5%–20% sucrose in either phosphate-buffered saline or water. In general, large quantities of DNA have been used for injection, with a linear dose responsiveness seen, at least in skeletal muscle, between 10 and 100 μg of DNA (Wolff et al. 1990). Similarly, it appears that the success of the injection technique is independent of the type of anesthesia used and the method of ventilation.

Taken together, these studies have provided us with complementary quantitative and qualitative data concerning recombinant gene expression in the heart. Specifically, they have addressed the following important questions: (a) What cells can be induced to express recombinant genes following injection of DNA? (b) How efficient is recombinant gene expression? (c) How stable is recombinant gene expression? (d) Can recombinant genes introduced into cardiac myocytes be regulated in response to humoral signals? In an initial set of studies, Leiden and coworkers (Lin et al. 1990) injected the pRSV–βgal plasmid (see Figure 1) directly into the apical portion of the beating left ventricular wall of 6- to 11-week-old rat hearts. Rats were killed either 3–5 days or 3–4 weeks following injection, and sections of the injected hearts were stained for β-galactosidase activity. These studies demonstrated recombinant β-galactosidase activity in >75% of injected hearts at both 3–5 days and 3–4 weeks following injection (Figure 2). β-Galactosidase was detected histochemically only in cardiac myocytes (and not in fibroblasts or vascular cells) and only in the area of DNA injection. Recombinant gene expression was a relatively low-frequency event, occurring in <1/100 myocytes in the area of injection. However, the absolute levels of β-galactosidase expression were not quantified in these studies. In control experiments, no β-galactosidase activity was detected in rat hearts injected with either vehicle alone or an irrelevant control plasmid. Recent follow-up studies have shown that β-galactosidase can still be detected 5–6 months following injection of pRSV–βgal into 6-week-old rat hearts (E. Barr and J. Leiden, unpublished

observations, 1991). Finally, histologic examination of the injected hearts demonstrated an acute inflammatory response in the area of injection at 3–4 days that often evolved into a fibrous scar by 4 weeks following injection. This inflammatory reaction, which was composed of both mononuclear and polymorphonuclear leukocytes, did not appear to represent a response to the injected DNA, as it was also observed following injection of vehicle (5% sucrose in PBS).

In a second set of studies, Leinwand and coworkers (Kitsis et al. 1991) injected the pRSV–CAT and pαMHC–Luc (Figure 1) plasmids into the left ventricular wall of Sprague–Dawley rats and assayed homogenates of the injected hearts for CAT and luciferase activity 5 days following injection. In addition, they injected the same plasmids into rat skeletal muscle (adductor magnus) to compare the efficiency of recombinant gene expression in cardiac and skeletal myocytes. These experiments demonstrated recombinant gene expression in 100% of the hearts and 78% of the skeletal muscles of injected animals. Interestingly, rat hearts expressed 10–100 times more CAT activity per microgram of protein lysate than skeletal muscle injected with the same amount of pRSV–CAT DNA (Figure 3). The reason for this difference remains obscure, but could be due to differences in the efficiency of DNA uptake or expression. CAT and luciferase activities were detected in 100% of the hearts at 14 days and 40% of the hearts at 60 days (P. Buttrick, M. Kaplan, and L. Leinwand, unpublished observations, 1991). In contemplating this approach to gene therapy, one consideration is the quantity of gene product produced by the injected gene. Use of the sensitive luciferase reporter gene enabled quantification of the amount of recombinant gene product being produced in the heart. The RSV promoter led to the production of 400–600 pg of luciferase activity per heart 5–14 days after injection.

In a third study, Wolff and coworkers (Acsadi et al. 1991) extended their original observations concerning recombinant gene expression in skeletal muscle fibers to cardiac muscle. Specifically, they showed that β-galactosidase, luciferase, and CAT genes under the control of the RSV LTR were expressed in cardiac myocytes following injection into

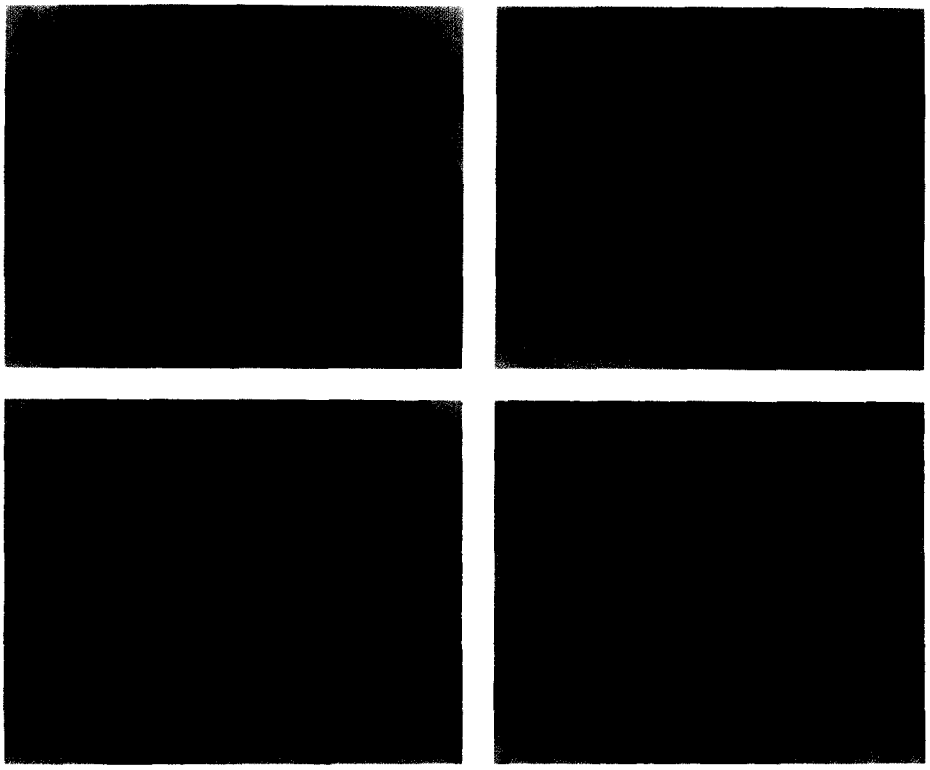
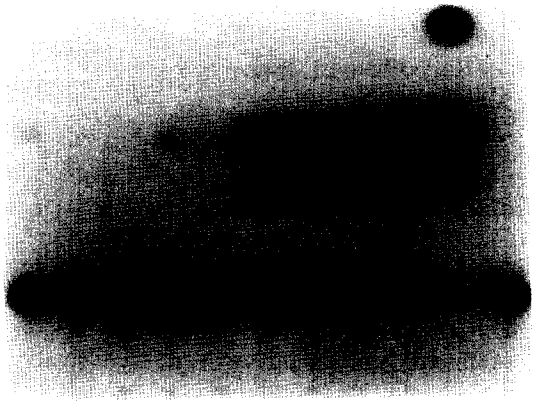


Figure 2. Expression of the recombinant β-galactosidase (βgal) gene in cardiac myocytes in vivo following direct injection of pRSV (Rous sarcoma virus)–βgal DNA into the left ventricular wall; 100 μg of pRSV–βgal DNA was injected into the beating apical wall of the left ventricle of Sprague–Dawley rats with use of a 30-gauge needle. Hearts were harvested 3–5 days or 3–4 weeks after injection and stained for β-galactosidase activity. **(A)** 10× view of a 3-mm section of a heart 3 days after pRSV–βgal injection. **(B)** 18× view of a 3-mm section from a heart 27 days after pRSV–βgal injection. **(C and D)** 125× and 250× views, respectively, of 4-μm sections from a heart 3 days after pRSV–βgal injection. β-Galactosidase activity (*dark blue staining*) is seen only within cardiac myocytes, which can be identified by their myofibrillar architecture. From Lin et al. (1990).

Figure 3. Heart and skeletal muscles express injected genes. Chloramphenicol acetyltransferase (CAT) activities in rat skeletal muscles (*S*) and hearts (*H*) coinjected with 100 μg each of pRSV–CAT and pα cardiac myosin heavy-chain (MHC)–luciferase (Luc) are shown; 20% of each organ homogenate was assayed 5 days after DNA injection. The above autoradiogram resulted from a 12-h exposure. Percent chloramphenicol conversion is indicated below each lane. *Plus and minus* correspond to positive and negative controls consisting of homogenization buffer with or without partially purified *Escherichia coli* CAT. From Kitsis et al. (1991).



the beating left ventricular wall of adult Sprague–Dawley rats. In contrast to the results reported by Leiden and Leinwand, Wolff's group found that CAT and luciferase expression was detectable for <25 days after injection. Because recombinant gene expression could be stabilized for at least 60 days by immunosuppression of the injected rats with cyclosporin A, they attributed the observed instability of expression to an immune response against the recombinant gene products. This hypothesis was further supported by the finding that luciferase expression also appeared to be stable for at least 60 days in nude rat hearts injected with the pRSV–Luc plasmid. The reason for the differences in the stability of recombinant gene expression observed by Wolff's group as compared with the results of Leinwand and Leiden remains unclear. However, Wolff and coworkers injected plasmid DNA in normal saline while both Leinwand and Leiden injected DNA in PBS–sucrose.

An important concern with all models of gene therapy is the potential for damage to host cells by integration of recombinant genes into the host genome. To address this issue directly, Acsadi et al. (1991) used Southern-blot analyses to demonstrate that the bulk of the recombinant DNA in cardiac myocytes from the injected hearts is retained in closed circular and linear forms and has not been integrated into the host genome. Although small amounts of integrated DNA probably would not be detected by use of these approaches, this finding is important because it suggests that this method of introducing recombinant genes into cardiac myocytes may not pose a significant risk of mutagenesis of the recipient cells.

• Regulation of Recombinant Genes in Cardiac Myocytes

The ability to achieve gene transfer through direct DNA injection offers a straightforward means of studying gene regulation in vivo. This approach is clearly simpler and less costly than the generation of transgenic animals, which has traditionally been the only means of studying the response of gene sequences to complex physiologic stimuli. The study of cardiac gene regulation has been largely restricted to the transfection of cultured fetal or neonatal cardiomyocytes. While useful, these studies have been limited by two factors. First, cardiac myocytes do not divide in culture. Second, it is not possible to study the effects of complex physiologic phenomena such as hypertension in a cell culture environment.

To apply direct gene transfer to the study of cardiac gene regulation, it was necessary to demonstrate that an injected gene would be regulated appropriately. Experiments were designed to ask whether a cardiac-specific promoter would direct expression of a reporter gene in a tissue-restricted and hormonally responsive manner (Kitsis et al. 1991). The rat αMHC promoter was chosen for these studies because the expression of this gene in vivo is restricted to the heart and is positively regulated by thyroid hormone. Upstream of the αMHC gene, 613 base pairs of DNA were linked to the luciferase reporter gene to produce the pαMHC–Luc plasmid (Figure 1), which was injected into both cardiac and skeletal muscle. In these experiments, organs were always coinjected with the pRSV–CAT reference plasmid as an internal control. Luciferase activity was then normalized to CAT activity to control for animal-to-animal variability as well as for known differences in transfection efficiencies between cardiac and skeletal muscle (see Figure 3). Luciferase activity was detected at high levels in heart, but not in skeletal muscle. Therefore, expression of an injected gene driven by a cellular promoter was regulated in the manner of the endogenous myosin heavy-chain gene.

To address the ability to modulate the expression of an injected gene, the influence of thyroid hormone on the expression of injected pαMHC–Luc was studied. αMHC mRNA and protein are positively regulated by thyroid hormone in vivo, and a construct bearing the same 5′ flanking region used in the injections has been shown to confer thyroid hormone responsiveness to a reporter gene transfected into fetal cardiac myocytes (Tsika et al. 1990). However, the regulatory elements of this gene have not been mapped in vivo, nor has it been possible to assess the thyroid responsiveness of DNA constructs in the adult heart. To assess the ability of pαMHC–Luc to respond to thyroid hormone, animals were made hypothyroid and then hyperthyroid, and their hearts were coinjected with pαMHC–Luc and pRSV–CAT. Of note, CAT activity from the injected RSV–CAT plasmid was significantly depressed in hyperthyroid relative to euthyroid and hypothyroid animals. Since the RSV promoter is not known to be responsive to thyroid hormone, this observation points to the need to include reference plasmids to control for variations in the transfection efficiency of organs in different physiologic states. The mean Luc/CAT ratio of hyperthyroid animals was 2–3 times greater than that of euthyroid or hypothyroid animals (Figure 4). These results, coupled with the tissue-restricted expression of the injected pαMHC–Luc plasmid, strongly suggested that expression of injected genes can be targeted to cardiac myocytes by use of a cardiac-specific promoter, and that their expression can be modulated by the hormonal status of the animal.

• Summary and Future Directions

It is clear from the studies summarized above that recombinant genes can be introduced into and expressed in cardiac myocytes following direct injection of plasmid DNA into the left ventricular wall of rodents. Although the methods employed and conclusions reached by the three laboratories involved in this work vary slightly, the results are most remarkable for their consistency and reproducibility. Taken together, these studies have demonstrated (a) that directly injected recombinant DNA is taken up and expressed by a small percentage of cardiac myocytes in the area of injection, (b) that the level of recombinant gene expression in heart appears to be higher than that observed in skeletal muscle following similar DNA injections, and (c) that the injected genes appear to be regulated normally by humoral signals. The stability of recombinant gene expression following direct DNA injections remains controversial and may be effected by an immune response directed against the recombinant gene products. Finally, the ability to introduce recombinant genes into eukaryotic tissues in vivo by direct injection appears to be limited to skeletal and cardiac muscle (Acsadi et al. 1991). The molecular mechanisms that facilitate DNA uptake in striated muscle remain unclear, but the possibilities

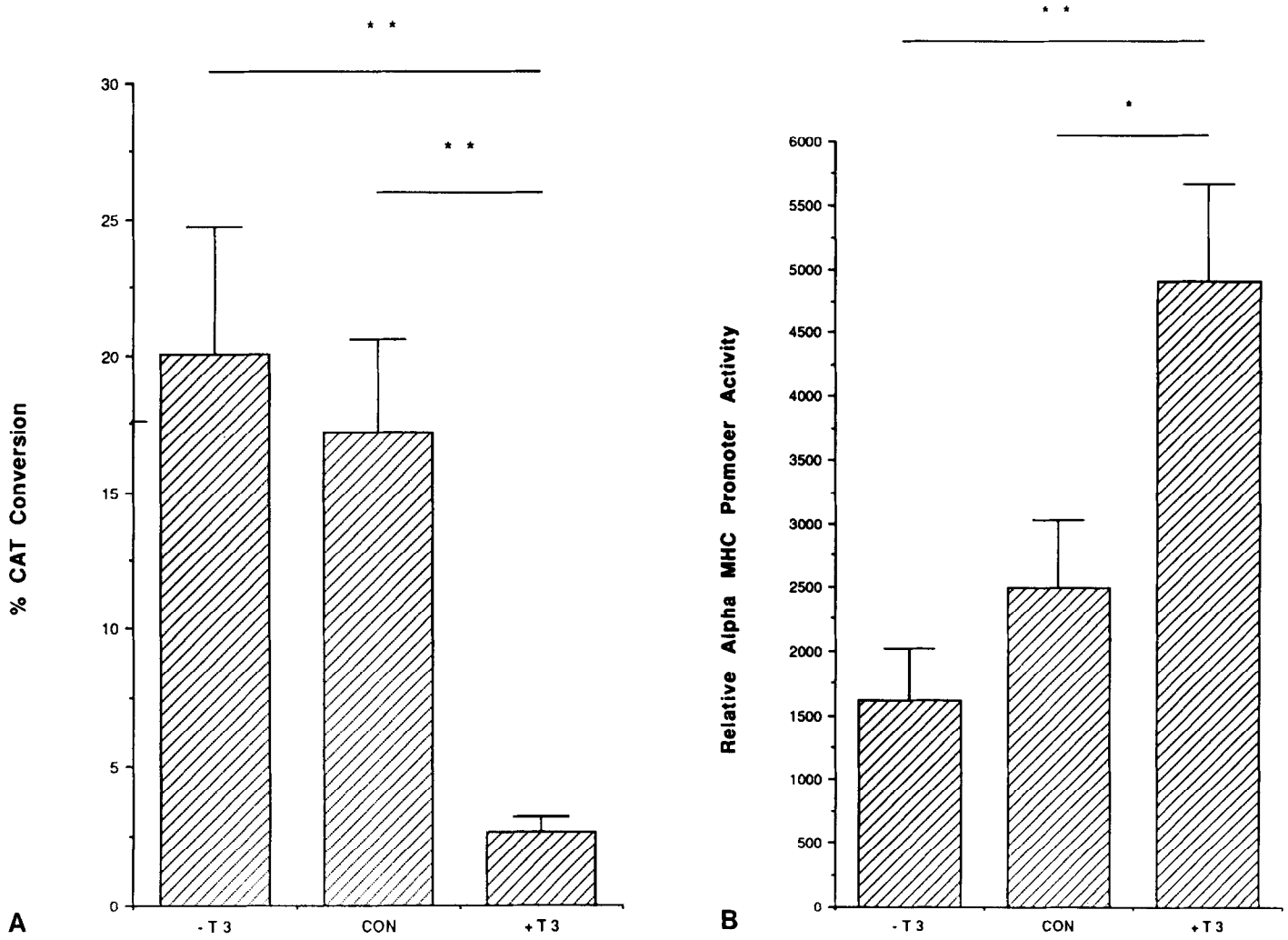


Figure 4. Thyroid hormone modulation of the expression of a gene injected into heart muscle. Activities of CAT and luciferase in hearts coinjected with pαMHC–Luc and pRSV–CAT from thyroid-hormone-manipulated animals. **(A and B)** CAT activities (% conversion; 15% homogenate) and Luc/CAT ratios [raw luminometer units; 5% homogenate]/[% conversion; 15% homogenate] in hearts from hypothyroid animals ($-T^3$) (n=6), hypothyroid animals treated with T^3 ($+T^3$) (n=6), and euthyroid controls (CON) (n=5). An *asterisk* indicates a significance value: *p<0.05 and **p<0.005. Data are expressed as mean ± SEM. From Kitsis et al. (1991).

include specialized striated muscle transport systems such as the T tubules or the unique ability to disrupt the sarcolemma transiently during recombinant DNA injections.

Regardless of the underlying mechanism, direct injection of plasmid DNA has several unique advantages as compared with other previously described methods of somatic gene therapy. First, the direct-injection method obviates the need for viral vectors with their concomitant risk of persistent infection of the recipient host. Secondly, it appears that the injected DNA is taken up and retained by myocytes in an episomal (that is, nonintegrated) form. Thus, the risk of mutagenesis of the recipient cells may be minimized, particularly as compared with retroviral vectors that usually integrate into the host genome. Finally, this method allows for the introduction of recombinant genes directly into cardiac myocytes in vivo, without the need for prior isolation and manipulation in tissue culture, a requirement that would render transfection of cardiac myocytes particularly difficult.

Direct injection of recombinant DNA into the myocardium holds promise for the treatment of a number of inherited and acquired diseases of the cardiovascular system. At least three types of applications are currently being explored. First, experiments are under way to express recombinant angiogenesis factors in cardiac myocytes in an attempt to stimulate collateral circulation in areas of ischemic myocardium (E. Barr, G. Englemann, and J. Leiden, unpublished results, 1991). This type of application has the advantage of not necessarily requiring recombinant gene expression by large numbers of cardiac myocytes. The technique is also being explored as a method of treating inherited defects of cardiac muscle proteins such as Duchenne's muscular dystrophy (abnormal dystrophin expression) and familial hypertrophic cardiomyopathy (abnormal βMHC expression). Finally, this method might be employed to replace circulating proteins in patients with inherited serum protein deficiencies. Recent preliminary studies have demonstrated that low levels of circulating human growth hormone (0.15 ng/ml) can be produced in rats following direct injection of 100 μg of a plasmid containing the human growth hormone gene under the control

of the RSV LTR (E. Barr and J. Leiden, unpublished results, 1991).

Apart from its potential therapeutic applications, the DNA injection technique represents a powerful tool for basic studies of cardiac transcription. Such studies have been limited in the past by the lack of a stable and transfectable cardiac cell line, and the substantial expense and time involved in using transgenic systems to study transcriptional regulation in the heart. These studies can now be carried out in 3–5 days after injection of appropriate reporter constructs into rodent myocardium. Moreover, the method represents a simple and economical way to test the promoter and enhancer elements of transgenic constructs prior to embryo injections.

Although the direct-injection method described in this review represents the first step toward somatic gene therapy in the heart, a number of questions and problems must be addressed before it becomes a viable therapeutic modality. First, it must be determined that human myocytes, like their rodent counterparts, can take up and express injected DNA. In addition, the stability of recombinant gene expression must be investigated more fully, with particular emphasis on the relationships between injection technique, recipient species, and different recombinant proteins, and the potential immune and inflammatory responses evoked by DNA injection. Finally, the current injection methods must be modified in an attempt to increase the efficiency of recombinant gene transduction and to decrease the inflammatory response following DNA injection. Potentially useful changes include alterations in the injection solution, the use of DC shock prior to or following DNA injection, the induction of transient ischemia at the time of injection, the use of cardiac perfusion with DNA-containing solutions as an alternative to direct injection, and the use of local and/or systemic antiinflammatory agents. Although each of these problems represents a substantial technical and experimental challenge, the initial success with direct injection of DNA into myocardium suggests that this technique may eventually lead to novel approaches for the therapy of cardiovascular disease.

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